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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*
In the first year we have demonstrated rapid initiation of m ammary metaplasia upon tissue specific stabilization of β-catenin, made fundamental observations on the role of the thymus and antigen specific regulatory T cells in suppressing active immune response against a defined antigen, and have embarked on improving tissue specific expression of our model antigen. Transgenic mice expressing low levels of HA, harbored antigen specific regulatory CD4 T cells. This was largely due to intra-thymic expression of this antigen. Adoptive T cell transfer experiments demonstrated that these cells proliferate as extensively as naïve $CD4^+$ T cells upon immunization, without losing their phenotypic characteristics. Regulatory T cells eventually dominated the response expressing mostly IL-10 but not IL-2 or IFN-γ. Thus, the capacity of suppressive T cells to expand upon antigen encounter is an essential component of imm une regulation *in vivo*. These observations support the view that depletion or de-programing of regulatory cells prior to immunotherapy can be beneficial, in controlling cancer. We are currently pursuing vaccination strategies that could reverse the state of tolerance, by deprogramming tolerance.

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Table of Contents

INTRODUCTION: Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

Autoimmune diseases represent a failure of self-tolerance to a particular tissue, and thus exemplify the counterpart of what is desirable in tumor immunotherapy. An understanding of the breakdown of self-tolerance in the course of tissue-specific autoimmunity will yield important insights pertaining to effective tumor specific immune responses and control of cancer. The growth of mammary tumors reflect the partial or total compromise of tumor specific immune surveillance in the tumor bearing host. By following the function and fate of T cells that recognize a defined antigen expressed by autochtonously arising mammary cancer, it is possible to define the mechanisms involved in immune evasion by the tumor, and to open possibilities for targeted therapy. To this aim animal models of inducible breast cancer in mice are being developed. In the currently available model, targeted Cre-lox-P mediated genetic recombination is used to remove exon 3 of the beta catenin gene, and produce a mutant stabilized molecule. At the same time when Cre is activated, a surrogate tumor antigen is activated by genetic recombination. The surrogate tumor-specific antigen (influenza hemagglutinin) has been chosen in order to make use of T cell receptor transgenic mice with class II and class I MHC restricted TCRs for influenza hemagglutinin. This animal model enables us to study in previously unknown detail the homing, activation and/or tolerization of naïve T cells in a setting when breast cancer develops.

BODY: This section of the report shall describe the research accomplishments associated with each task outlined in the **approved** Statement Of Work.

Task 1: To express the influenza HA as a neo-self antigen in the mammary alveolar cells *in vivo*.

We have taken advantage of the Cre/loxP recombination system to construct a mouse with ubiquitous but inducible expression of influenza HA. A mini-gene cassette was constructed in which the PGK promoter drives expression of a floxed lacZ gene, and upon activation of Cre it's expression is replaced by that of HA (Figure 1). From 12 founders, one showed ubiquitous expression of the cassette. Several observations indicate that expression is low but sufficient to elicit an immune response. HA staining of tissue sections, or western blotting of mammary extracts from transgenic animals after Cre mediated recombination did not reveal detectable levels of HA, while DNA PCR analysis of tissues expressing Cre revealed recombination and deletion of the lacZ-stop cDNA insert.

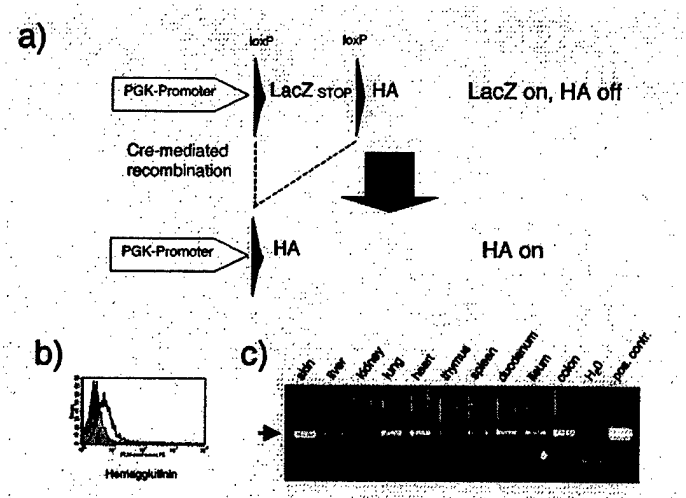

Figure 1: Characterization of the PGK LacZ HA transgenic mouse. a) Schematic representation of the PGK LacZ HA construct. Cre mediated recombination leads to excision of LacZ, thus leading to transcription of a translatable HA coding region. b) Verification of HA-expression in transfectants by FACS analysis. The transgenic construct was transfected into 293T cells with (open histogram) or without (grey shaded histogram) co-transfection of a Cre-expressing vector. Expression of Cre leads to expression of HA, as detected by surface staining with the HA-specific mAb 37.38. c) Ubiquitous expression of the transgene in [PGK ^{loxP} LacZ ^{loxP} HA] transgenic mice. Expression of the transgenic construct was assessed by RT-PCR of RNA prepared from the indicated organs using primers located in the LacZ coding region. Control PCR in the absence of RT did not yield signals (not shown).

The ($PGK^{loxP}lacZ^{loxP}HA$) mice were crossed to mice expressing an HA-specific transgenic T cell receptor on CD4 T cells (TCR6.5). The lymphoid phenotype of ($PGK^{loxP}lacZ^{loxP}HA$

x WAPCre x TCR6.5) compound mutant mice was compared to control-littermates that were either (TCR6.5) simple mutant or (PGK^{loxP}lacZ^{loxP}HA x TCR6.5) compound transgenic mice. All the mice were F4 or higher back-crosses to the H-2d (BALB/c) haplotype. FACS analysis of expression of cell surface markers by the lymphocytes revealed that the single and double transgenic mice were identical with respect to thymic cellularity and frequency of TCR6.5 positive cells among CD4$^+$ T cells, both in the thymus, lymph nodes, and spleen. This indicated the tight control of the switch cassette and the absence of HA expression in (PGK^{loxP}lacZ ^{loxP}HA) mice, in the absence of Cre-mediated recombination (Figure 2).
By contrast, the phenotype of triple transgenic (PGK^{loxP}lacZ^{loxP}HA x WAPCre x TCR6.5) mice was dramatically different. Thymic cellularity was diminished by about 50 Among thymic CD4$^+$ single-positives, TCR6.5+ cells were reduced from 38% to 16%, totaling in a four-fold reduction of 6.5+ CD4+ T-cells (Figure 3, a). In mesenteric lymph nodes and spleen the proportion of TCR-6.5 expressing cells among CD4 T cells was very small (2% versus 12% in controls) (Figure 3, b). These results indicate that HA is expressed and is detected by the immune system.

Partial depletion of HA specific T cells was also observed in the lymph nodes and spleen. This unexpected widespread loss of HA specific T cells prompted a re-screening of the double transgenic WAP-Cre x PGK^{loxP}lacZ^{loxP}HA mice for tissue specific recombination and antigen expression. We have already documented mammary specific recombination driven by Cre in crosses of the WAP-Cre mice to R26R or Catnb$^{lox(ex3)}$mice, both in the submitted proposal and in a recently published report. It was therefore surprising to find germ line recombination in nearly all WAP-Cre x PGK^{loxP}lacZ^{loxP}HA double transgenic mice. A most plausible explanation is differences in relative susceptibilities of different genetic loci to Cre/loxP mediated recombination. Presumeably, in our founder line the HA cassette must have inserted in a locus that is more prone to Cre mediated recombination than the ROSA-26 locus.
To overcome the problem, and improve on the design, we took advantage of our

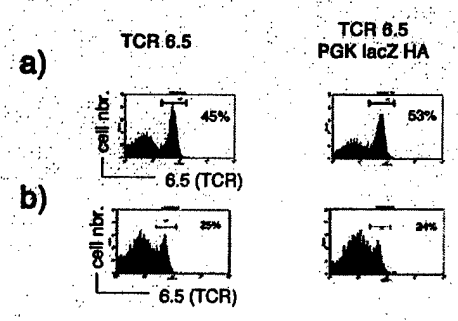

Figure 2: FACS-analysis of thymocytes of (PGK^{loxP}lacZ^{loxP}HA x TCR6.5) double transgenic animals. Proportions of CD4 and CD8 subsets were not severely affected in double-transgenics (not shown). Gating on CD4 single-positives revealed no change in the fraction of TCR6.5 positive cells in the thymus (a) or spleen (b).

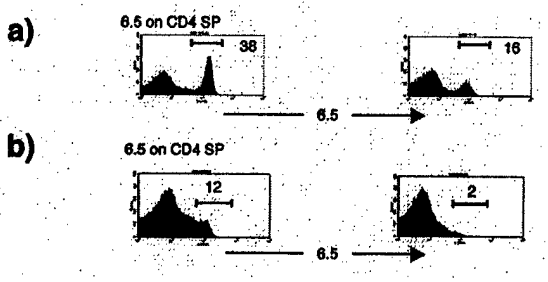

Fig. 3 TCR-6.5 CD4 T cells from the thymus and periphery of TCR-6.5 transgenic animals expressing HA. Thymus (a) or lymph nodes (b) of a TCR-6.5 single-transgenic (left) or a TCR-6.5 mouse expressing HA (right) were stained for CD4, and TCR-6.5 (anti clonotypic antibody 6.5). In the normal TCR-6.5 mouse, about 40% of CD4 single-positives thymocytes and approx. 12% of peripheral CD4 T cells express high levels of the transgenic TCR. In triple-transgenics, the proportion of cells expressing the transgenic TCR is reduced.

experience with the R26R mice (Figure 3) to generate two novel knock-in mice. In one case, the HA cassette is inserted in the ROSA-26 locus, to generate a mouse analogous to the R26R mouse (Figure 4, a). In the other case, a hormone inducible Cre, generated from the fusion of Cre and a mutant ligand binding domain of the human estrogen receptor (ERT2), is incorporated within the cassette (Figure 4, b). CreERT2 is activated by short term feeding of mice with tamoxifen (1 mg per mouse for up to 5 days), and allows for transient activation of Cre independent of lactation. Furthermore, HA was fused to a natural mouse tumor antigen (P1A) to allow comparison of immune responses against the two antigens. Currently, we are screening ES cell clones harboring the first construct, for homologous recombination. Recombinant (knock-in) mice will be generated from this construct.

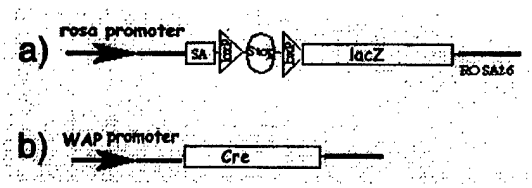

Figure 4 Schematic representation of the a) R26R, and b) WAP-Cre loci. In R26R, a transcription stop signal is flanked by loxP sequences, and the cassette is driven by the ROSA promoter. Rectangles represent loxP sites, and arrows indicate promoters.

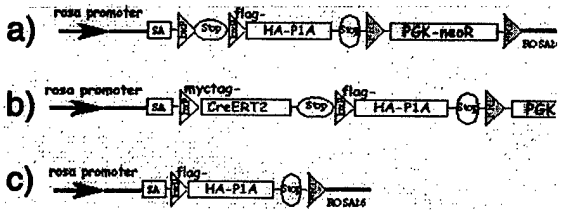

Figure 5 Schematic representation of the knock-in of (a) HA-P1A and, (b) Cre-ER-HA-P1A cassettes in the ROSA-26 locus. (c) Both cassettes recombine in the presence of Cre to express HA-P1A. In the unrecombined cassettes, multiple transcription and translation stop signals are flanked by loxP sequences; a splice acceptor signal allows splicing of the transgenic mRNA to the untranslated first exon of the ROSA gene. Expression of the transgenes are driven by the ROSA-26 gene promoter. Rectangles represent loxP sites, and arrow indicates promoter.

Task 2: To predictably and reproducibly initiate mammary tumors expressing HA in mice.

Mammary specific stabilization of β-catenin and rapid initiation of mammary lesions in an inducible manner was reported by us in Proc Nat Acad Sci USA (2002) 99: 219-224.

Task 3: To investigate the immunological consequences of neo-self antigen/surrogate tumor antigen.

The exact mechanisms compromising specific immune responses in cancer patients are likely to differ from tissue to tissue and the type of cell affected. A common denominator in all cancers is likely to be the promiscuous expression of tissue and tumor specific genes in the thymus. Recent observations in transgenic mice suggest promiscuous expression in the thymus of particular tumor antigens (1). One consequence of this may

be the intrathymic generation of regulatory T cells that suppress immune responses against tissue/tumor specific antigens (2); for reviews see (3-5). Indeed, adoptive T cell transfer together with myeoablative treatment have provided promising results in the treatment of melanoma patients (6), supporting the view that depletion of regulatory cells prior to immunotherapy can be beneficial, in controlling cancer.

Genomic PCR on DNA from triple transgenic mice for the recombined HA-locus confirmed that the loss of HA specific $CD4^+6.5^+$ T-cells (Figure 3) was indeed related to the germ-line recombination of the HA cassette, and therefore thymic expression of HA. To reveal the significance of low level intra thymic expression of HA on the host anti-HA immune response, we took advantage of such mice with ubiquitous low level expression of HA, henceforth referred to as pgk-HA.

As already described, double transgenic TCR6.5 x pgk-HA showed partial deletion of $CD4^+6.5^+$ T-cells (Figure 3). The majority (65-67%) of the remaining $CD4^+TCR6.5^+$ cells expressed Interleukin-2 (IL-2) receptor α-chain (CD25) (Figure 6, a &b). The $CD25^+$ thymocytes were anergic and suppressive as were the $CD25^+$ cells in the spleen (Figure 6, c). Interestingly, the $CD25^-$ TCR6.5 expressing cells in the spleen were not anergic and did not suppress the proliferative response of naïve TCR6.5 cells from TCR6.5 transgenic $RAG^{-/-}$ mice. These observations suggested intrathymic generation of HA specific regulatory T cells, as reported in other mouse models (7-9).

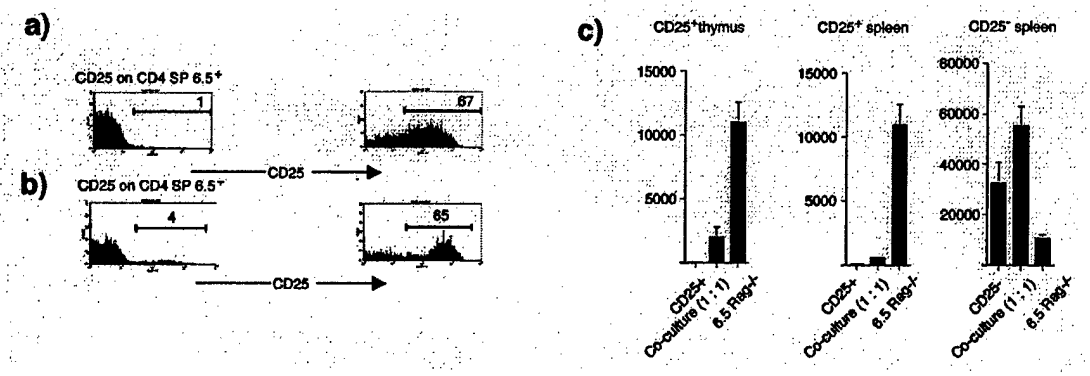

Figure 6. TCR-6.5 CD4 T cells from the thymus and periphery of TCR-6.5 x pgk-HA transgenic animals. Thymus (a) or lymph nodes (b) of a TCR-6.5 single-transgenic (left) or a TCR-6.5 mouse expressing HA (right) were stained for CD4, and TCR-6.5 (anti clonotypic antibody 6.5). In contrast to control thymocytes (TCR-6.5 single transgenics), almost 70% of $CD4^+$ 6.5^+ cells in the compound transgenic thymus expressing HA showed upregulation of the activation marker CD25 (IL2 receptor-α subunit). In lymph nodes (b) (and spleen, not shown), the reduction of $CD4^+6.5^+$ T cells was more pronounced than in the thymus. Again, about 2/3 of the 6.5^+ T cells express CD25. (c) $CD25^+$ cells from thymus (left) and spleen (middle) are anergic and inhibit the proliferation of naïve T cells *in vitro*. CD25 negative cells from spleen behave like naïve T cells, i.e. they proliferate vigorously in response to peptide. For proliferation assays, triplicates of 2×10^4 sorted cells from double transgenic animals or single transgenic TCR-6.5 $Rag^{-/-}$ mice were incubated with 2×10^5 irradiated BALB/c splenocytes in the presence of 10μg/ml HA peptide 107-119. After 90 hours, 1μCi of ^{3}H-Thymidin was added, and the cultures were harvested for scintillation counting after another 20 hours. Co-culture inhibition assays were performed at a ratio of 1 : 1. The residual peripheral $CD25^-$ 6.5^{int} cells from *pgk-HA x TCR-6.5* mice were not anergic and did not suppress in the co-culture assay (Note the different scale of the y-axis in the right bar diagram).

Properties of antigen specific regulatory T cells are still a matter of debate. Several characteristics of these cells have emerged from *in vitro* studies (reviewed in (10) resulting in the notion that regulatory T cells are anergic in terms of proliferation, suppress other cells by direct cell contact which requires neither IL-10 nor TGF-β and which results in the inability of suppressed $CD4^+$ T cells to produce IL-2 (11-13). However, some observations underline that it is at present unclear in how far these observations *in vitro* are in fact a reflection of the properties of $CD4^+CD25^+$ T cells *in vivo*. First, in some experimental systems of immune regulation by $CD4^+CD25^+$ T cells *in vivo* it was found that soluble factors such as IL-4, IL-10 and TGF-β do contribute to the prevention of autoimmunity, with the role of these factors varying between different models (14-16). Second, polyclonal $CD25^+CD4^+$ T cells proliferate and expand when they are transferred into $rag^{-/-}$ or IL-2 receptor β deficient mice (17-19), indicating that their anergic state is reversible. We therefore took advantage of the regulatory T cells generated in our double transgenic mice to address their proliferative response to antigen *in vivo*, and to extrapolate from such studies possible mechanism of immune suppression by such cells.

The relatively high frequency of antigen specific $CD25^+$ regulatory T cells in *pgk-HA x TCR-6.5* mice allowed for the isolation of sufficient numbers of these cells to perform adoptive transfer experiments. The allotypic marker Thy1 was used to distinguish between donor derived (Thy1.2) and host cells (Thy1.1). In initial experiments, we determined that the frequency of donor derived cells among host cells was similar (0.03-0.05% of CD4 T cells) when equal numbers (3×10^5) of either 6.5^+CD25^+ cells isolated from *pgk-HA x TCR-6.5* mice or naïve 6.5^+CD25^- cells from *TCR-HA* $rag^{-/-}$ mice were transferred into wild-type hosts (not shown). Fourteen days after transfer of 6.5^+CD25^+ cells, the majority (> 85%) of donor derived cells still expressed the CD25 marker (not shown). In order to address whether the transferred cells remained functionally stable, we isolated the donor-derived population after they had been parked in antigen free hosts for six days (Figure 7).

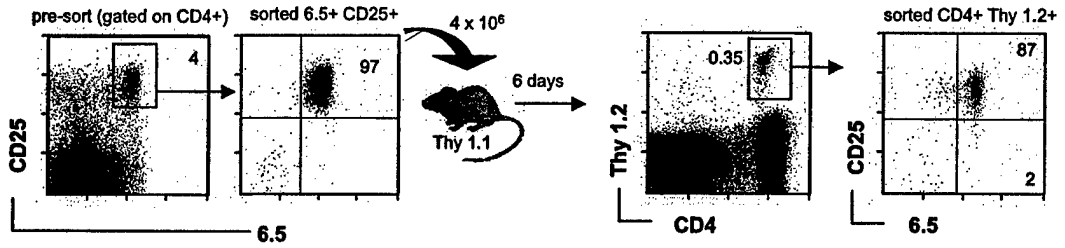

Fig. 7. Purified $CD4^+CD25^+6.5^+$ T cells (4×10^6) from $Thy1.2^{+/+}$ *pgk-HA x TCR-6.5* mice were transferred into BALB/c $Thy1.1^{+/+}$ mice. The sorting gate and a representative analysis of the purity of cells after sorting is shown in the left dot plots. Six days after transfer recipients were sacrificed. Peripheral lymph nodes and spleen were pooled and stained for CD4, Thy 1.2, CD25 and 6.5. The frequency of donor derived cells among CD4 T cells is shown together with the sorting-gate used for re-isolation of cells. The right dot plot shows the purity of re-isolated cells.

Re-isolated cells were completely anergic and efficiently suppressed the proliferation of naïve 6.5^+ CD4 T cells (Figure 8, a). The same result was obtained with $CD25^+$ regulatory

T cells obtained from pgk-HA thymus grafted mice where expression of antigen in peripheral lymphoid organs could be excluded (not shown).
We next asked whether adoptively transferred 6.5^+CD25^+ cells could inhibit the response of endogenous HA-specific T cells. Wild-type mice that had received 3 x 10^5 6.5^+CD25^+ cells, resulting in a frequency of approximately 1 in 3000 $CD4^+$ T cells, or untreated control mice were immunized with $HA^{107-119}$ peptide in incomplete Freund's adjuvant (IFA). Eight days after the immunization cells from the draining lymph nodes were restimulated *in vitro* (Figure 8, b). While cells from control animals proliferated vigorously, we did not observe any proliferation with cells from mice that had received

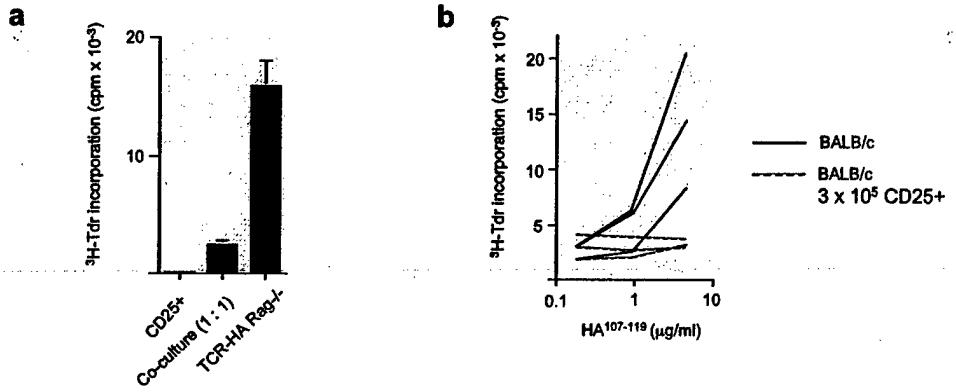

Fig. 8. (A) Re-isolated $Thy1.2^{+/+}$ cells six days after transfer were tested for their proliferative response upon stimulation with HA-peptide and suppressive potential when co-cultured with naïve cells as described in Figure 6. (B) Recipients of 3 x 10^5 purified $CD4^+CD25^+6.5^+$ T cells (dashed lines) or untreated BALB/c mice (solid lines) were immunized with HA-peptide (100µg) in incomplete Freund's adjuvant. Eight days later, draining lymph node cells were harvested and stimulated *in vitro* with titrated amounts of HA-peptide for 90 hours. Incorporation of ^{3}H-thymidin within the last 20 hours was measured by scintillation counting. The graph shows the data for three immunized mice of each group representative for three independent experiments.

regulatory T cells.
To examine the proliferative response of regulatory T cells to antigen *in vivo* we next performed co-transfer experiments with sorted 6.5^+CD25^+ cells from *pgk-HA x TCR-6.5* mice and naïve 6.5^+CD25^- cells from *TCR-6.5* $rag^{-/-}$ mice into Thy1.1 BALB/c mice. Donor derived cells were followed by means of the Thy1.2 marker. One control group was injected with 3 x 10^5 6.5^+CD25^- cells only and immunized as before. Analysis of draining lymph node cells on day eight after immunization revealed that ca. 4% of CD4 T cells were donor derived. By contrast, in distant lymphoid compartments such as mesenteric lymph node and spleen less than 0.05% of CD4 T cells were Thy 1.2 positive, similar to the values observed in non-immunized recipients. Thus, HA-specific 'naïve' T cells appeared to have expanded at least 100-fold in the antigen exposed lymph node. A second group of animals received 3 x 10^5 6.5^+CD25^+ cells and was immunized and analyzed as above. On the contrary to what might have been expected in light of the *in vitro* 'anergy' of these cells, we found a distribution of donor derived cells in these animals very similar to that observed in recipients of naïve cells. Thus, the draining lymph nodes contained ca. 3% of donor derived cells among CD4 T cells, while in other

secondary lymphoid compartments less than 0.05% of CD4 T cells were Thy1.2$^+$. A third group of animals received both 6.5$^+$CD25$^+$ cells and 6.5$^+$CD25$^-$ cells. In these animals, the frequency of donor derived cells in the draining lymph node eight days after immunization again was approximately 3% of CD4 T cells. To confirm that the transferred regulatory T cells maintained their phenotype, we re-stimulated draining lymph node cells derived from the three groups of immunized animals with HA-peptide *in vitro*. While recipients of naïve cells showed a very strong proliferative response neither recipients of 6.5$^+$CD25$^+$ cells nor recipients of mixed populations displayed any antigen specific *in vitro* proliferation.

In order to verify that the increased numbers of 6.5$^+$CD25$^+$ cells in the antigen exposed lymph nodes was a result of proliferation rather then homing and/or survival, we labeled 6.5$^+$CD25$^+$ cells with CFSE prior to adoptive transfer. A second group of mice received CFSE labeled naïve 6.5$^+$CD25$^-$ cells. Recipient animals were then immunized as before

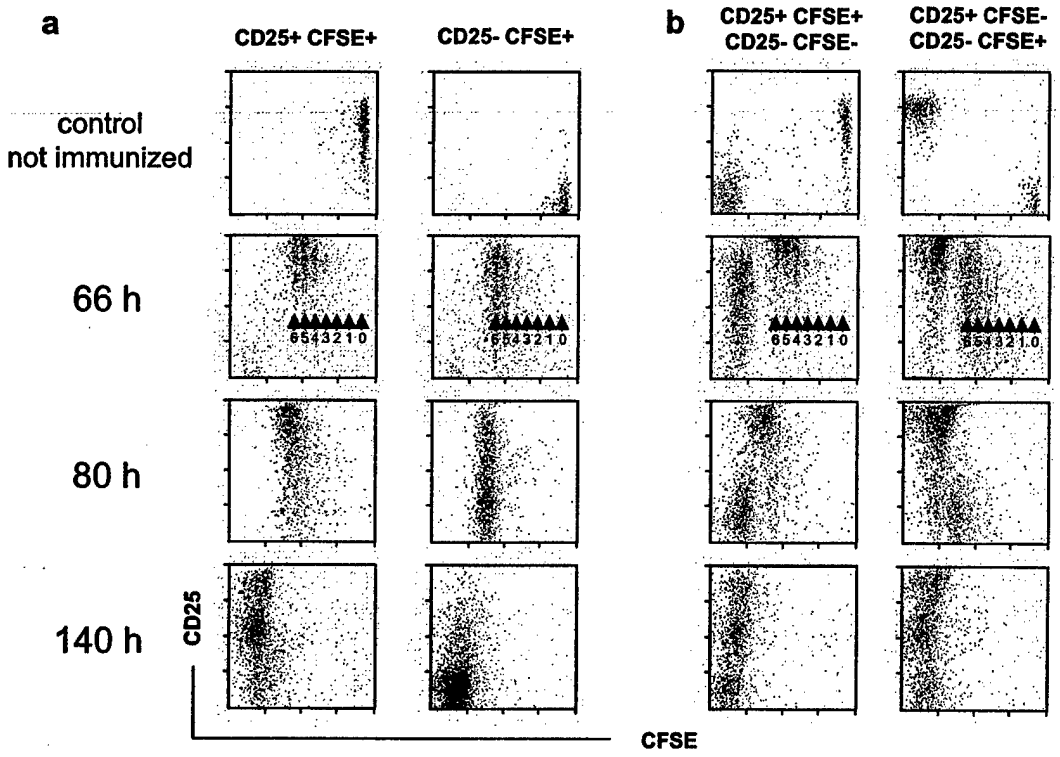

Fig.9. Proliferation of adoptively transferred CD4$^+$CD25$^+$6.5$^+$ T cells or CD4$^+$CD25$^-$6.5$^+$ T cells in the draining lymph nodes after immunization

(a) 3 x 10^5 CFSE-labeled CD4$^+$CD25$^+$6.5$^+$ T cells from *pgk-HA x TCR-6.5* mice (left) or CFSE-labeled naive CD4$^+$CD25$^-$6.5$^+$ T cells from *TCR-6.5 rag-/-* mice were transferred into BALB/c Thy1.1 recipients. Two days later, recipients were immunized with 100 μg HA-peptide in IFA. Controls were immunized with IFA without peptide. Mice were sacrificed at the indicated time points after immunization and draining lymph node cells were harvested and stained for CD4, Thy1.2 and CD25. The dot plots show the expression of CD25 versus CFSE fluorescence intensity on gated donor-derived cells (CD4$^+$Thy1.2$^+$). Numbered arrows within the dot plots (66 hr) indicate the number of divisions of CFSE-labeled cells. (b) 3 x 10^5 CFSE-labeled CD4$^+$CD25$^+$6.5$^+$ T cells from *pgk-HA x TCR-6.5* mice and 3 x 10^5 naive unlabeled CD4$^+$CD25$^-$6.5$^+$ T cells from *TC-R6.5 rag-/-* mice (left) or 3 x 10^5 CFSE-labeled naive CD4$^+$CD25$^-$6.5$^+$ T cells from *TCR-6.5 rag-/-* mice and 3 x 10^5 unlabeled CD4$^+$CD25$^+$6.5$^+$ T cells from *pgk-HA x TCR-6.5* mice (right) were transferred into BALB/c Thy1.1 recipients. Mice were immunized and analyzed as in (a).

and the phenotype of donor derived cells in the draining lymph nodes was followed in a kinetic fashion by gating on $CD4^+Thy1.2^+$ cells. As early as 66 hours after immunization, the majority of donor derived cells in the draining lymph nodes of both groups of animals had cycled more than four times (Figure 9a). The progeny of 6.5^+CD25^+ cells had further up-regulated the CD25 marker, and at this point in time CD25 was now also expressed by the progeny of 6.5^+CD25^- cells. Eighty hours after immunization, cells in both groups of animals had undergone further divisions, with the exact division number no longer being discernable. All together, these observations indicate that *in vivo* both regulatory and naïve T cells proliferate in response to antigen.
At this point we addressed the important question of how regulatory cells suppress an active antigen specific immune response. Two possibilities were considered: That regulatory cell influence antigen encountered naïve cells by altering their phenotype and function, or their expansion and contraction (death or cell cycle).

Measurements of intracellular cytokines established that in the simultaneous presence of regulatory and naïve T cells of identical specificity the expanded pool of donor derived CD4 T cells displayed a cytokine profile closely resembling the one observed in the exclusive presence of expanded regulatory T cells. However, since in these experiments both naïve and regulatory T cells were of Thy1.2 origin, they did not allow for a conclusive assessment of whether this was due to a dominant expansion of the regulatory T cells in the co-transfer, or whether eventually the progeny of naïve T cells may have adopted an altered pattern of cytokine secretion ('immune deviation'), and thus would have contributed to the prevalence of IL-10 producing cells. In order to distinguish between these two possibilities, we determined the cytokine production eight days after immunization by $Thy1.1^+$ CD4 T cells T in the two groups of mice described in either normally expanded or 'suppressed' progeny of naïve $CD25^-$ cells. As shown in Figure 10, the fraction among these cells of producers of IL-2, IL-10, IFNγ or TNFα was not significantly affected by the presence of regulatory T cells, indicating that 'immune

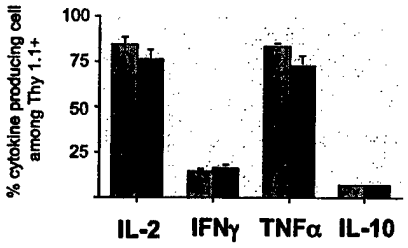

Fig. 10 cytokine production upon immunization of adoptively transferred naïve $CD4^+CD25^-6.5^+$ T cells in the presence or absence of $CD4^+CD25^+6.5^+$ regulatory T cells. 5 x 10^5 CFSE labeled naïve $CD4^+CD25^-6.5^+$ T cells sorted from $Thy1.1^+$ *TCR-HA* mice ($rag^{+/+}$) were adoptively transferred into BALB/c Thy1.2 recipients (left) or BALB/c Thy1.2 recipients that had in addition received an equal number of $CD4^+CD25^+6.5^+$ T cells from Thy1.2 from *pgk-HA x TCR-6.5* mice. Draining lymph node cells of recipient animals were harvested on day 8 after immunization with IFA and peptide, stimulated *in vitro* with PMA/Ionomycin for 6 hr in the presence of Brefeldin A and stained for CD4, Thy1.1 and the respective cytokine as indicated. The frequency of cytokine producing cells among gated $Thy1.1^+CD4^+$ T cells is shown. Grey bars correspond to recipients of naïve $CD4^+CD25^-6.5^+$ T cells alone, black bars correspond to recipients of naïve $CD4^+CD25^-6.5^+$ T cells and $CD4^+CD25^+6.5^+$ regulatory T cells (mean of 4 per group).

deviation' was not the mechanism by which regulatory cells influenced normal antigen specific T cells.

In order to more conclusively visualize the influence of regulatory T cells on the homeostasis of $CD25^-$ cells at different phases, we adoptively transferred CFSE-labeled $Thy1.1^+6.5^+CD25^-$ T cells into two groups of Thy1.2 recipients that had either received $Thy1.2^+6.5^+CD25^+$ regulatory T cells or not. Gating on $Thy1.1^+$ CD4 T cells was then used to visualize and quantify the expansion of the naïve T cells after immunization. The initial recruitment of cells into the response, their proliferation rate and the early expansion up to 90 hours after the immunization was almost identical in both groups (Figure 11). At 90 hours after the immunization, however, the $Thy1.1^+$ T cells ceased to expand in those animals that had received regulatory T cells, and a steady decline in the fraction of $Thy1.1^+$ cells among CD4 T cells set in.

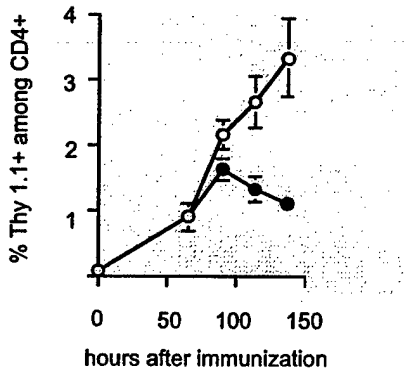

Fig. 11 Frequency in the draining lymph nodes of the progeny of naïve $CD4^+CD25^-6.5^+$ T cells upon immunization in the presence (open circles) or absence (closed circles) of $CD4^+CD25^+6.5^+$regulatory T cells. Draining lymph node cells of recipient animals treated as in Figure 10 were harvested at the indicated time points and stained for CD4 and Thy1.1. The frequency of $Thy1.1^+$ cells among $CD4^+$ T cells is shown (mean of 4 per group). The frequency at 0 hr (i.e., without immunization) was 0.05 % of CD4 T cells.

The data presented here indicate that *in vivo* an expanding population of regulatory T cells suppress the response of naïve T cells, in a somewhat unexpected fashion, in that the regulators 'overgrow' the progeny of $CD25^-$ cells while the initial recruitment into cycling and the early expansion of these cells is apparently not affected. It remains to be shown how regulatory cells exert their influence on other cells in vivo, and how different modes of vaccination affect the kinetics of proliferation of one or the other T cell population. Importantly, our studies so far show how thymic expression of an antigen may determine the outcome of immune response against an antigen expressed in the a peripheral tissue or tumor.

The new transgenic mice under construction will allow us to confirm and extend these studies in the mammary tumor model.

KEY RESEARCH ACCOMPLISHMENTS:

- Crosses of WAP-Cre to R26R (that encode a Cre dependent lacZ in the ROSA26 locus) resulted in mammary specific expression of lacZ.
- Crosses of WAP-Cre or MMTV-Cre mice with $Catnb^{lox(ex3)}$mice resulted in mammary specific lesions and extensive induction of metaplasia in 100% of double transgenic mice.
- $PGK^{loxP}lacZ^{loxP}HA$ transgenic mice were generated that expressed an ectopic antigen (influenza HA) in a Cre/loxP manner and were crossed with WAP-Cre mice.
- In contrast to $Catnb^{lox(ex3)}$or R26R mice, Cre/loxP mediated recombination in $PGK^{loxP}lacZ^{loxP}HA$ transgenic mice was not tissue specific but rather involved the germ line; this indicates that the frequency of recombination is strictly locus dependent.
- To achieve mammary specific Cre/loxP dependent expression of HA, new knock-in mice encoding the HA cassette in the ROSA-26 locus were designed and production of mice are in progress.
- Expression of HA in the recombined $PGK^{loxP}lacZ^{loxP}HA$ mice led to the appearance of potent regulatory T cells, that suppressed active immune responses in an antigen dependent manner.
- Low level intra-thymic expression of HA was sufficient for the appearance of HA specific regulatory T cells; this observation has direct implications for anti tumor immune responses, as many tumor and tissue specific antigens are known to be expressed in low levels in the thymus.
- Adoptive transfer experiments using such regulatory T cells provided the unexpected result that the cells are (contrary to expectations) not anergic *in vivo*, but rather expand in response to vaccination to dominate the immune response to antigen.
- The mode of action of such regulatory T cells seems to be primarily to suppress the expansion of naïve T cells upon encounter of antigen. There was no evidence for immune deviation in the naïve T cell population.
- The above results need to be confirmed and extended in the new mice with mammary and tumor specific expression of HA.

REPORTABLE OUTCOMES:

The following two manuscripts have been submitted:

Dynamics of immune regulation *in vivo* by antigen-specific $CD25^{+}CD4^{+}$ suppressor T cells. Ludger Klein, Khashayarsha Khazaie, and Harald von Boehmer[1]

Gene transfer and genetic modification of ES cells by Cre and Cre-PR expressing MESV based retroviral vectors. Stelios Psarras, Niki Karagianni, Christoph Kellendonk, François Tronche, François-Loic Cosset, Carol Stocking, Volker Schirrmacher, Harald von Boehmer and Khashayarsha Khazaie.

CONCLUSIONS:

An important scientific outcome of this work so far is the finding that low level intrathymic expression of HA leads not only to partial deletion but also to the positive selection of HA specific regulatory T cells. Any active therapeutic strategy in mammary cancer should consider the likelihood of presence of such cells in the patients. In agreement with this notion, adoptive T cell transfer together with myeoablative treatment have provided promising results in the treatment of melanoma patients (6), supporting the view that depletion of regulatory cells prior to immunotherapy can be beneficial, in controlling cancer.

Understanding the mechanism of function of regulatory T cells will help to design successful therapeutic intervention in the disease. Our work provided the unexpected result that the HA specific regulatory T cells are (contrary to expectations) not anergic *in vivo*, but rather expand in response to vaccination to dominate the immune response to antigen. The mode of action of such regulatory T cells seems to be primarily to suppress the expansion of naïve T cells upon encounter of antigen, rather than induce immune deviation in the naïve T cell population. While these results need to be confirmed and extended in the new mice with mammary and tumor specific expression of HA, they strongly indicate that vaccination attempts for correcting immune deviation late in disease may be futile. Instead, efforts should be made to eradicate the expanded regulatory T cell pool and/or to change their properties. Here, combinations of adoptive T cell therapy and vaccination may be a promising avenue.

We have learned important technical lessons from the Cre/loxP based animal models generated so far in the course of this work. The choice of genetic locus for targeted recombination is critical. It appears that certain loci are significantly more susceptible than others for recombination. These are unsuitable for engineering tissue/tumor specific recombination, as they tend to promote germ-line recombination events. One suitable locus is the ROSA-26. Cre mediated recombination in this site is tightly controlled and when Cre is expressed in a tissue specific manner, the resulting recombination is tissue specific. We have therefore embarked on making knock-in mice, inserting our HA cassette in this locus.

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